Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 4050

www.rsc.org/obc

PAPER

Searching for new cell-penetrating agents: hybrid cyclobutane–proline γ,γ-peptides†

Esther Gorrea,[a] Daniel Carbajo,[b,c] Raquel Gutiérrez-Abad,[a] Ona Illa,[a] Vicenç Branchadell,[a] Miriam Royo[b,c] and Rosa M. Ortuño*[a]

Received 30th January 2012, Accepted 2nd April 2012
DOI: 10.1039/c2ob25220a

Two generations of hybrid γ,γ-peptides containing cyclobutane amino acids and *cis*-γ-amino-L-proline joined in alternation have been synthesized and their capacity to cross the eukaryotic cell membrane has been evaluated. The first generation consists of di-, tetra- and hexapeptides, and their properties have been analyzed as well as the influence of peptide length and chirality of the cyclobutane residues. Results have shown that the absolute configuration of the cyclobutane amino acid does not have a relevant influence. The second generation consists of hybrid γ,γ-hexapeptides with a common backbone and distinct side chains introduced with different linkage types through the α-amino group (N^{α}) of the proline monomers. These peptides have been shown to be non-toxic towards HeLa cells and to internalize them effectively, the best results being obtained for the peptides with a spacer of five carbons between the N^{α} atom and the guanidinium group. The introduction of cyclobutane residues inside the sequence affords a good balance between charge and hydrophobicity, reducing the number of positive charges. This results in lower toxicity and similar cell-uptake properties when compared to previously described peptide agents.

Introduction

The use of natural peptides as therapeutic agents in living organisms has been often restricted due to their low stability, especially against proteases. The fact that peptides could adopt stable helical structures, turns, or β-sheet-like structures gave the starting point for the design of peptidomimetics of natural proteins with related functions. In particular, β- and γ-peptides are stable in water and to enzymatic degradation *in vitro* and *in vivo*,[1] which appears to be an important advantage compared to natural peptides. This property has implications for pharmaceutical use, as they may be active by oral administration and could present better bioavailability than the peptide drugs. Therefore, within some of the most important properties described so far for β-peptides, the inhibition of the fat and cholesterol absorption, the antimicrobial activity and the ability to penetrate the cell membrane can be emphasized.[2] Examples for γ-peptides are scarcer but point in the same direction.[3]

In recent years, the use of peptides as drug carriers has been one of the most explored applications. Since the discovery of peptide sequences capable of translocating cell membrane in the late 80s, cell-penetrating peptides (CPPs)[4] have been demonstrated to be a good alternative to other drug transporter systems such as viral delivery agents,[5] liposomes,[6] encapsulation in polymers,[7] or electroporation,[8] which often have not shown sufficiently good efficiency, in addition to causing high cellular toxicities in some cases. Moreover, some of these methods are restricted to *in vitro* applications.

However, several negative aspects that limit the use of α-peptides in living organisms have also been described, such as the rapid enzymatic degradation, the sometimes low permeability of the cell membrane and the high toxicity of some of these peptides.[9] The fact that α-CCPs can easily be degraded implies that the doses to be administrated to obtain a positive response have to be higher, dramatically increasing their toxicity. Thus, different methods to improve resistance to proteases have been considered,[10] such as the use of peptidomimetics containing D-α-amino acids, β-peptides, γ-peptides, peptoids or combinations of those.

Most CPPs described in the literature are natural peptides derived from peptide sequences responsible for cellular internalization of membrane proteins or proteins that cross the cell membrane. TAT peptide[11] has been used as a reference when preparing, studying and analyzing the properties of different new peptides as possible cell-penetrating agents.

[a]*Departament de Química, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain. E-mail: rosa.ortuno@uab.es; Fax: +(34) 935811265*
[b]*Combinatorial Chemistry Unit, Barcelona Science Park, University of Barcelona, Baldiri Reixac, 10, 08028 Barcelona, Spain*
[c]*CIBER-BBN, Networking Centre on Bioengineering, Biomaterials, and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain*

†Electronic supplementary information (ESI) available: Full details on experimental procedures, product characterization and complementary biological tests. See DOI: 10.1039/c2ob25220a

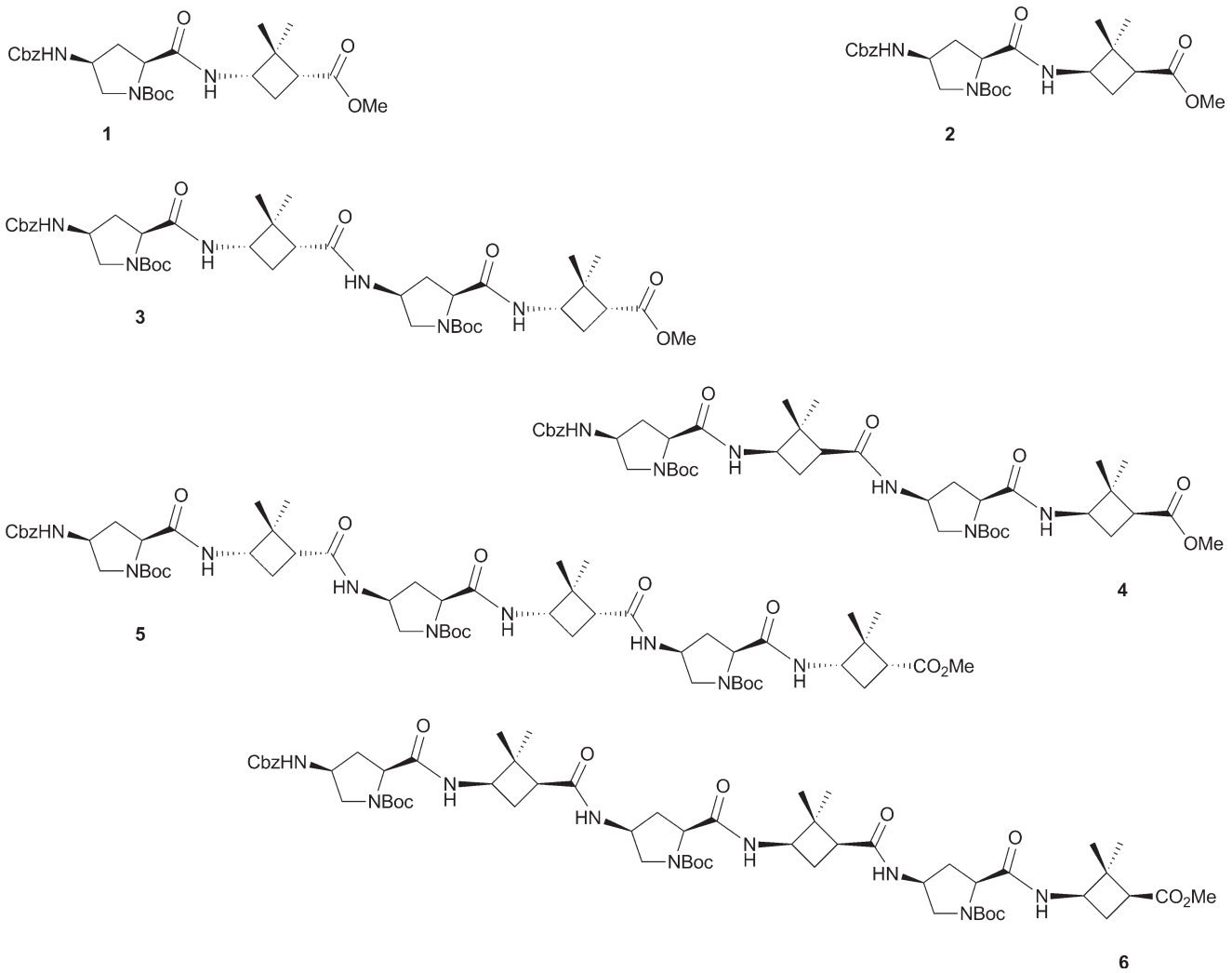


Chart 1 Structures of the first generation of hybrid cyclobutane–proline γ,γ-peptides.

One of the examples of CPPs is the use of proline derivatives incorporated in peptide structures. It has been reported that proline-rich peptides[12] and proline dendrimers[13] can be internalized by eukaryotic cells. In this context, a synthetic method for the preparation of conformationally constrained γ-peptides incorporating γ-amino-L-proline or derivatives was described.[14,15] The ability of these γ-peptides to enter into different cell lines (COS-1 and HeLa) *via* an endocytic mechanism was demonstrated and these agents were shown to offer advantages over the well-known penetrating TAT peptide, such as being less toxic than TAT and protease resistance. In all cases, the side chain hydrophobicity determined the cell-uptake properties, the elongation of alkyl chains and the presence of polar or cationic groups being very important factors to improve their penetration activities.[15]

Our research group has achieved stereoselective synthetic strategies for obtaining unnatural β-[16] and γ-amino acids[17] and peptides including a cyclobutane moiety as constrictor of the peptide backbone, which, at the same time, can induce specific folded structures. Some of these compounds have shown biological activity as metallocarboxypeptidase inhibitors.[18] The constricted cyclobutane moieties used in our laboratory caught our attention as possible units to be inserted between the proline derivatives, in order to prepare new CPPs. Thus, in previous work, orthogonally protected amino acids derived from (−)-verbenone were used for the preparation of hybrid γ,γ-peptides, by coupling with a *cis*-4-amino-L-proline derivative. A first generation of hybrid cyclobutane–proline γ,γ-peptides of different size and with modifications of the chirality of the cyclobutane residue was prepared (Chart 1). The synthesis of those compounds was achieved by using synthetic strategies in solution, commonly applied in our laboratory. In addition, their ability to fold and tendency to aggregate were then studied by microscopic techniques, NMR experiments and computational calculations.[19]

In the present work, we provide the results of the screening of peptides **1–6** as CPPs towards HeLa cells by flow cytometry. Based on the preliminary results obtained, a second generation of hybrid γ,γ-hexapeptides was designed and synthesized. In these compounds, different side chains were introduced with different linkage types through the α-amino (N^{α}) group of the proline monomers. Inspired by earlier work,[15] three different γ,γ-hexapeptide families were synthesized, N^{α}-acyl-γ,γ-hexapeptides (polyamides on the side chains), N^{α}-alkyl-γ,γ-hexapeptides (polyamines on the side chains), and N^{α}-guanidylated-γ,γ-hexapeptides. The cell-uptake properties of these new compounds

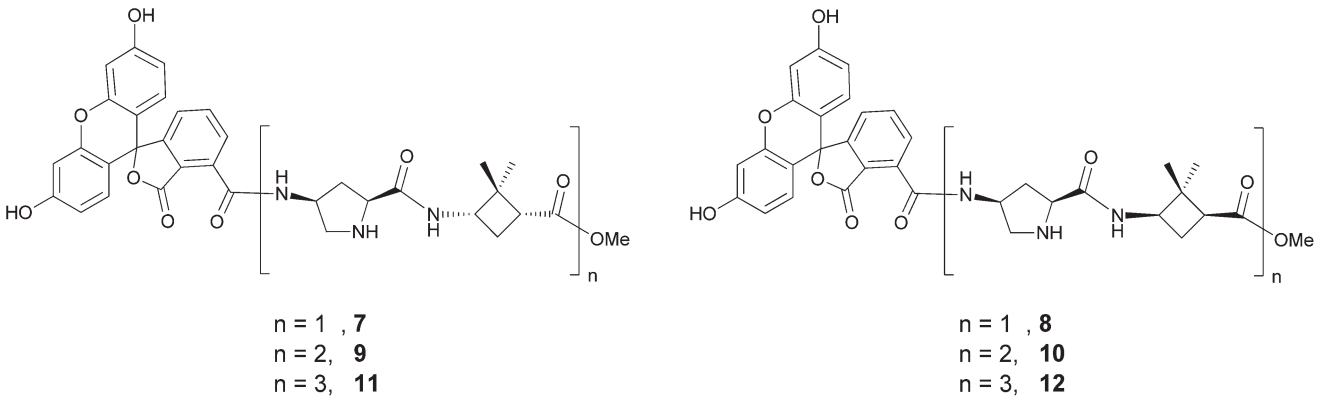
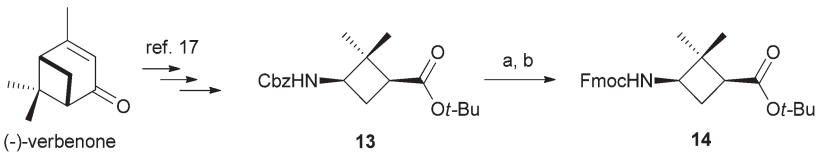
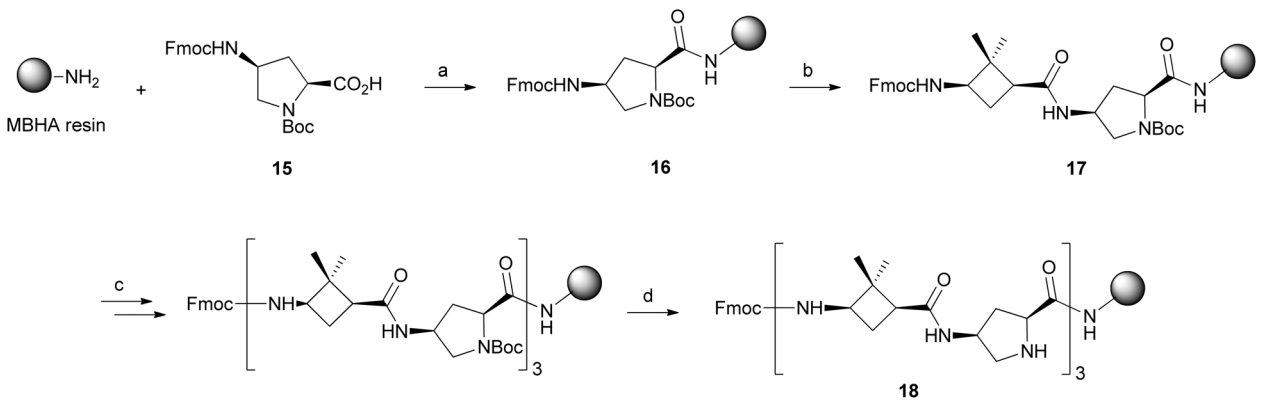


Chart 2 Structures of the first family of hybrid cyclobutane–proline γ,γ-peptides labeled with 5(6)-carboxyfluorescein.



Scheme 1 *Reagents and conditions*: (a) H_2 (7–8 atm), 10% $Pd(OH)_2/C$, EtOAc, rt, o.n. (quantitative); (b) Fmoc-O-Su, acetone–H_2O, pH = 9, rt, o.n. (64%).



Scheme 2 *Reagents and conditions*: (a) HOBt, HBTU, DIPEA, DMF, rt, 2 h; (b) (1) piperidine, DMF; (2) deprotected acid **14**, HOBt, HBTU, DIPEA, DMF, rt, 2 h; (c) repetition of steps; (d) TFA, DCM.

were investigated taking into account the possible influence on the biological activity of factors such as the nature and length of carbon chains and the presence of additional functional groups. Their cytotoxicity towards HeLa cells was analyzed and their activity as CPPs was evaluated by using flow cytometry. The results are described herein.

Results and discussion

1 Synthesis of peptides

1.1 5(6)-Carboxyfluorescein (CF-OH) anchorage to the first generation of hybrid γ,γ-peptides. In previous work,[19] we described the synthesis in solution of the first generation of hybrid cyclobutane–proline γ,γ-peptides. In order to test them as CPPs, some transformations were carried out.

The N-terminal proline γ-amino group of di-, tetra-, and hexapeptides **1–6** was deprotected by hydrogenolysis and the fluorescent label 5(6)-carboxyfluorescein (CF) was introduced using PyBOP as a coupling agent. Subsequently, the N^{α}-Boc protecting group was removed by acidolysis with TFA. In this way, peptides **7–12** were obtained (Chart 2).

1.2 Solid-phase synthesis of the second generation of hybrid cyclobutane–proline γ,γ-hexapeptides. As stated before, some modifications of the N^{α}-side chain in proline residues can induce improvements in the cell penetration properties of the final oligomers.[14,15] Therefore, various γ,γ-hexapeptides based on a cyclobutane γ-amino acid and *cis*-γ-amino-L-proline (see Schemes 1 and 2) joined in alternation were synthesized to investigate their activities. These oligomers present a common backbone and bear distinct side chains that have been introduced with different linkage types through the α-amino group of the proline monomer. Based on the linkage type, three different peptide families were obtained and evaluated.

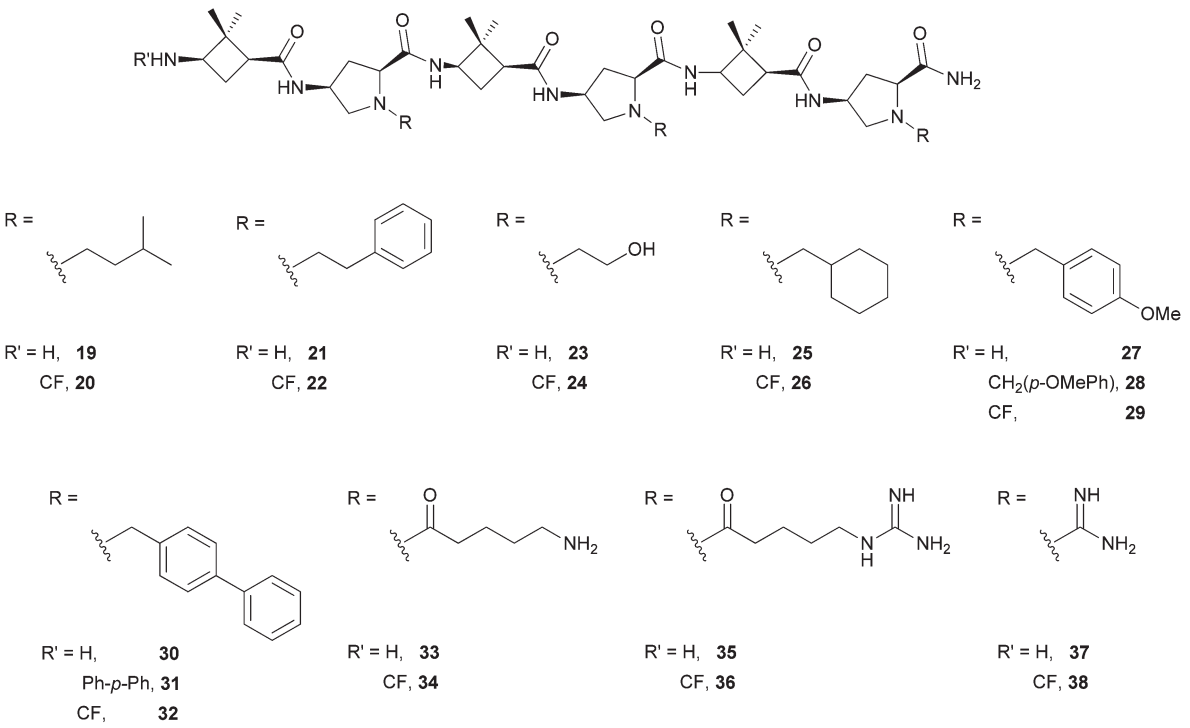


Chart 3 Chemical structure of the second generation of hybrid γ-γ-proline–cyclobutane hexapeptides synthesized.

The synthesis of the cyclobutane monomer was carried out in solution following a stereoselective strategy, which had previously been optimized in our laboratory.[17] From **13**, hydrogenolysis of benzyl carbamate using palladium hydroxide on charcoal as catalyst followed by reaction with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide in a mixture of acetone–water at pH = 9[20] led to Fmoc-protected amine **14** (Scheme 1).

Subsequently, the solid-phase synthesis of the γ,γ-hexapeptide backbone was carried out (Scheme 2). The use of solid-phase peptide-synthesis (SPPS) provides some advantages over the classical liquid-phase synthesis. The general principle of SPPS is the repetition of cycles of coupling–wash–deprotection–wash. Among the various protection schemes that have been developed, we chose the combination of the protecting groups 9-fluorenylmethyloxycarbonyl (Fmoc) and *tert*-butoxycarbonyl (Boc), based on the concept of orthogonal protection. The resin selected was methylbenzhydrylamine (MBHA) linker supported on polystyrene.

Commercially available (2*S*,4*S*)-4-(*N*-Fmoc)amino-1-(*N*-Boc) pyrrolidine-2-carboxylic acid, **15**, was used as the precursor of the proline moiety. This derivative was the first one to be anchored to the resin through amide bond formation, to afford **16** (see the Experimental section for details). HBTU was used as a coupling agent in the presence of HOBt as a catalyst and DIPEA. Next, the Fmoc protecting group was removed by reaction with piperidine in DMF. To proceed with the next stage of the synthesis, the *O*-Boc group in **14** was removed using TFA in DCM in a separate experiment. Straight afterwards, the free carboxylic acid obtained was coupled to the amino acid linked to the resin, **16**, to give the protected dipeptide **17** following the same procedure described above. From this stage, the formation of the hexapeptide skeleton was achieved by repeating twice more the last steps described. Then N^{α}-Boc was eliminated by treating the resin with TFA in DCM.

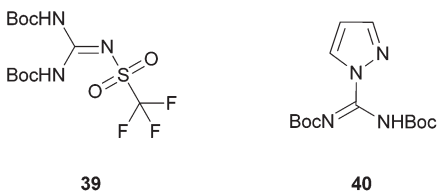


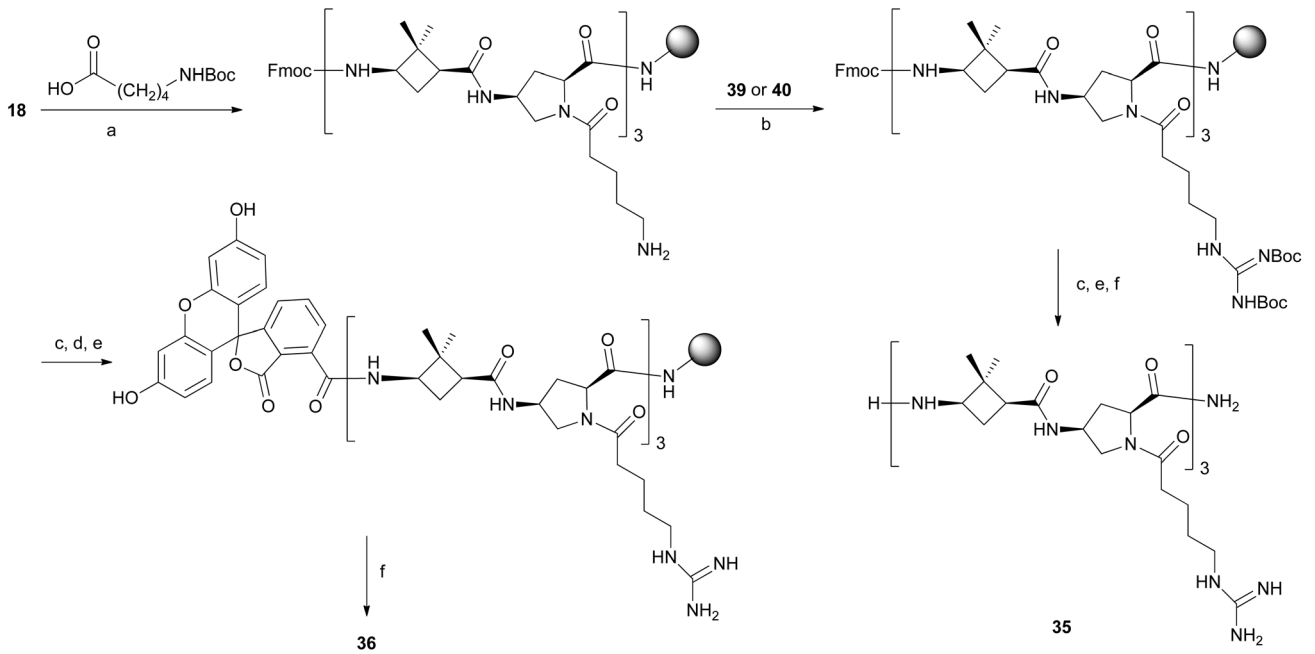
Chart 4 Guanidylation agents: *N*,*N*-diBoc-*N*′-trifluoromethanesulfonyl guanidine, **39**; *N*,*N*-diBoc-1*H*-pyrazole-1-carboxamide, **40**.

The next step was the introduction of N^{α}-side chains. N^{α}-Alkyl-γ,γ-peptides were prepared *via* reductive amination using the corresponding aldehyde followed by reduction with $NaBH_3(CN)$. In this way, precursors to **19–32** were obtained (Chart 3).

For N^{α}-acyl-γ,γ-peptides, precursors leading to **33–36** were synthesized by reaction of the corresponding deprotected γ,γ-hexapeptide with 5-(*N*-Boc-amino)valeric acid, using HBTU and HOBt as coupling agents. Boc removal from the newly attached chains led to the formation of free amines (**33** and **34** precursors).

For the synthesis of **35** and **36** precursors, the introduction of the guanidinium group was assayed by using two possible reagents, **39** and **40** (Chart 4), which have been described in the literature to guanidylate primary and secondary amines.[21] The guanidylation was carried out in the presence of Et_3N and in a DCM–DMF mixture as solvent. Reaction was complete after 24 h by using either reagent.

For N^{α}-guanidylated-γ,γ-peptides, the introduction of the guanidinium group only worked satisfactorily with **39**, although



Scheme 3 *Reagents and conditions*: (a) (1) HBTU, HOBt, DIPEA, DMF, rt; (2) TFA, DCM, rt; (b) Et_3N, DCM, rt; (c) piperidine, DCM, rt; (d) CF-OH, HBTU, HOBt, Et_3N; (e) TFA, DCM, rt; (f) HF, anisole, 0 °C, 1 h.

completion required eleven days reaction time. In this way, precursors of **37** and **38** were prepared.

Then, *N*-Fmoc protection was removed by using a 50% solution of piperidine in DMF. After cleavage from the resin by treatment with anhydrous HF, free γ,γ-hexapeptides **19**, **21**, **25**, **27**, **30**, **33**, **35** and **37** were obtained, purified by semi-preparative reverse-phase HPLC, and fully characterized (see ESI†).

Alternatively, after Fmoc removal, the CF moiety was introduced onto the N-terminal amino group employing HBTU, HOBt and DIPEA in DMF. The reaction was followed by piperidine washes just before cleavage of the peptide from the resin in order to remove over-incorporated CF.[22] CF-γ,γ-hexapeptides **20**, **22**, **24**, **26**, **29**, **34**, **36** and **38** were ultimately cleaved from the resin and purified to more than 95% homogeneity by HPLC. Their identity was verified by electro-spray and/or MALDI-TOF mass spectrometry (see ESI†).

Compounds **23** and **32** could not be satisfactorily purified because they co-eluted with impurities.

To illustrate all these processes, as an example, the synthesis of compounds **35** and **36** is depicted in Scheme 3.

It is worth mentioning that when introducing the side chains to obtain the **27** and **30** precursors, an undesired removal of the Fmoc N-terminal group followed by the introduction of an extra substituent was detected, thus obtaining **28** and **31** as well. This fact has previously been described.[23]

2 Cell-uptake evaluation

Firstly, the toxicity of γ,γ-peptides **7–12** was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Then, their cell-uptake properties were assayed by using HeLa cells by means of flow cytometry quantification techniques using TAT peptide as a reference (see ESI†).

All the compounds were shown to be non-toxic in the presence of the cells, the tetra (**9** and **10**) and hexapeptides (**11** and **12**) being less toxic than TAT peptide. Once their toxicity was tested, their ability to penetrate HeLa cells was studied at 37 °C by flow cytometry. Quantification by flow cytometry at pH 6 was performed to discriminate between effectively internalized peptides and membrane attached ones. Experiments carried out under these conditions with peptides **7–12** showed very low cell-uptake properties (see ESI†). The chirality of the cyclobutane moieties was shown to be not relevant for their penetration capacities.

After having studied the first generation of compounds, we redesigned and screened the new CF-γ,γ-hexapeptides shown in Chart 2. We considered oligomers consisting of six residues because previous results with γ-proline hexapeptides showed good cell-uptake properties.[15] As chirality was shown not to have an influence, the most synthetically accessible stereoisomer was chosen. Firstly, their toxicities towards HeLa cells were determined by the MTT assays using 25 μM solutions and at two different times (2 and 24 h) (Fig. 1 and ESI†). These were lower than that of TAT peptide, used as a reference, at the work concentration used in the cell-uptake preliminary screening.

The viability of HeLa cells after their treatment with the γ,γ-hexapeptides for 24 h was always higher than 95%. Only TAT exhibited slight cytotoxicity at 25 μM (85%) in HeLa cells.

The cell-uptake results (Fig. 2) show that the different peptides do not behave in the same way, indicating that the ability to cross the cell membrane depends on the structure and charge changes introduced by the N^{α}-side chains.

Thus, as observed in Fig. 2, γ,γ-hexapeptide **36** showed the highest increase on their cell-uptake properties. It presents

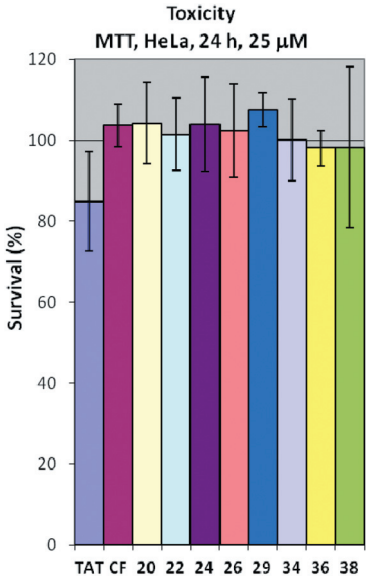


Fig. 1 Cytotoxicity of the different γ,γ-hexapeptides as monitored in HeLa cell lines. Cell death was quantified using the MTT assay after 24 h of incubation using 25 µM peptide concentration. Error bars represent standard deviation (SD) from the mean value of three independent experiments of each peptide.

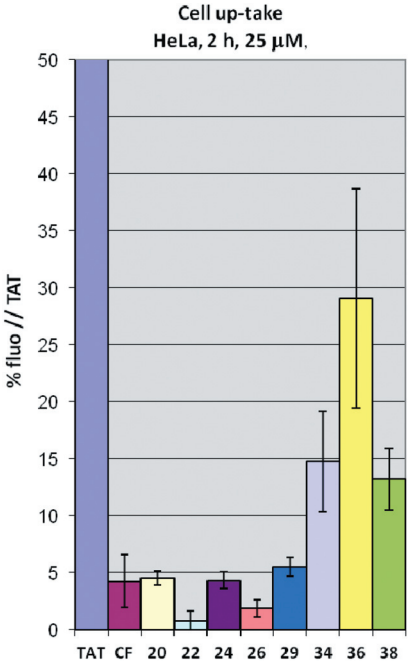


Fig. 2 Flow cytometry quantification of the cellular uptake of the different γ,γ-hexapeptides tested in HeLa cells. Cells were incubated with the peptide at a final concentration of 25 µM for 2 h at 37 °C. Error bars represent standard deviation (SD) from the mean value of three independent experiments of each peptide. The graph has been cut at 50% of fluorescence for clearness (TAT = 100%).

guanidinium groups at the side chains within its sequence, which in the cell environment would be protonated. The role of the spacer between the guanidinium group and the peptide backbone seems to be crucial, as the conformational flexibility and sterically unencumbered nature of the straight chain alkyl spacing groups have been reported to be important for efficient cellular uptake.[24] Probably, in γ,γ-hexapeptide **36**, the positive charge groups are more accessible to interact with the cell membrane than in N^{α}-guanidylated γ,γ-hexapeptide **38**, thus explaining the lower activity observed for the second compound. In addition, we realized that the presence of free primary amines on the side chains gave an extra advantage on the cell-penetrating properties, as shown by the results for **34**. In contrast, those molecules bearing highly hydrophobic carbon side-chains, **20**, **22**, **24**, **26**, and **29**, showed poor penetration activity compared with TAT.

Conclusions

Two generations of hybrid cyclobutane–proline γ,γ-peptides have been evaluated accounting for their cell-uptake properties. In view of preliminary results obtained on the first generation of previously described compounds, a second generation of new agents has been synthesized and screened. These oligomers present a common backbone and have distinct side chains introduced with different linkage types through the α-amino group of the proline monomer. Based on the linkage type, three different peptide families, namely N^{α}-acyl-γ,γ-hexapeptides, N^{α}-alkyl-γ,γ-hexapeptides, and N^{α}-guanidylated-γ,γ-hexapeptides, have been obtained. These new γ,γ-peptides combine a rigid and hydrophobic cyclobutane moiety and a proline derivative that provide hydrophilicity and diversity in the N^{α}-side chains. These oligomers were studied with the aim to find the optimal balance between hydrophobicity and positive charge.

The highest increase in the cell-uptake properties has been obtained for a hybrid γ,γ-hexapeptide in which the N^{α} atom of the proline residues is linked to a guanidinium group through a pentanoyl chain. The role of the spacer appears to be crucial for enhancing activity as deduced from comparison of results for peptides **36** and **38**. Probably, in the most active peptide **36**, guanidinium is more accessible for interaction with the cell membrane. In contrast, N^{α}-alkyl-γ,γ-hexapeptides bearing hydrophobic carbon side-chains, such as **20**, **22**, **24**, **26** and **29**, evidenced much lower penetration activity.

Differently to previously reported all-4-aminoproline γ-hexapeptides, in which six guanidinium groups are directly linked to the peptide backbone,[15] hybrid γ,γ-hexapeptide **36** presents only three guanidinium groups anchored to an N^{α}-alkyl chain. Therefore, charge balance is improved by the reduction of the total number of charges and the increase of hydrophobicity.[25] As a consequence, compound **36** presents similar uptake properties to those described for earlier peptides but lower toxicity.

Although the effectiveness of penetration revealed by the studied γ,γ-hexapeptides does not reach the level of some peptides described in the literature, their value as transporters could be guaranteed by establishing a favourable balance between their ability to penetrate, the capacity to accumulate inside different cellular organelles, and their low toxicity. Subcellular localization studies as well as synthesis of longer hybrid γ,γ-peptides are currently ongoing in our laboratories.

Experimental

MTT cytotoxicity assay

The viability of HeLa cells in the presence of the peptides was tested using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To avoid saturation in cell growth after 24 h of peptide incubation, 7×10^3 cells per well were seeded on a 96-well plate (Nange Nunc) for each assay. After 24 h, the culture medium was discarded and replaced by a new medium containing different CF-peptide concentrations. Cells were incubated for 2 h and 24 h at 37 °C under 5% CO_2 atmosphere, and MTT (0.5 mg mL^{-1}) was added 2 h before the end of incubation. After 2 h of incubation with MTT, the medium was discarded by aspiration and 2-propanol was added to dissolve formazan, a dark blue coloured crystal observed in the wells. Absorbance was measured at 570 nm in a spectrophotometric Elx800 Universal microplate reader (Bio-Tek), 30 min after the addition of 2-propanol. Cell viability is expressed as a percent ratio of cells treated with peptide to untreated cells, which were used as a control.

Flow cytometry

Flow cytometry was used to study the penetrating properties of the peptides. HeLa cells were seeded onto 35 mm plates at a concentration of 21.4×10^3 cells cm^{-2}. After 24 h, cells were then incubated for 2 h at 37 °C. After incubation time, cells were washed 3 times with PBS, detached with 0.25% trypsin–EDTA, centrifuged at 1000 × *g*, and washed again. To remove fluorescence of CF or CF-peptides bound to the plasma membrane, the pH of the PBS solution was brought down to 6 by the addition of 1 N HCl just before measuring fluorescence. At pH = 6, extracellular fluorescence of CF is quenched without altering cell mechanisms. Fluorescence analysis was performed with an Epics XL flow cytometer (Coulter). Triplicate analyses of each sample were performed for each condition, and results from independent experiments were normalized by subtraction of the auto fluorescence control value from each value and considering the value of TAT reference under the same experimental conditions as 100.

Synthetic procedures

For SPPS, commercially available methylbenzhydrylamine hydrochloride linker supported on polystyrene (with 0.63 mmol g^{-1} load) was used. So, after treating the resin for 20 min with TFA–DCM and for 2–3 min with DIPEA–DCM to remove HCl, the first amino acid can be attached to the resin using standard Fmoc coupling protocols.

The solid-phase synthesis of the γ,γ-hexapeptides was carried out choosing the proline derivative as the first unit to anchor to the resin. The coupling conditions were HBTU as coupling agent, with HOBt and DIPEA. Washing the resin with DCM eliminated any possible excess of reactants or reagents. Next, the Fmoc protecting group was removed by adding a 50% solution of piperidine in DMF. To proceed with the next stage of the synthesis, in a separate experiment the carboxyl group of the corresponding cyclobutane amino acid was deprotected using a 40% solution of TFA in DCM. Straight afterwards, the free acid obtained was coupled to the amino acid linked to the resin to afford the protected dipeptide following the same procedure described above. From this stage, the formation of the hexapeptide skeleton was achieved by repeating twice more the last steps described. To carry out the functionalization of the N^{α} amino groups, the secondary amine of the proline ring was deprotected by treating the resin with a 40% solution of TFA in DCM. The excess of TFA was eliminated by washing it with a 10% solution of DIPEA in DCM.

The synthetic procedures used to prepare the different peptides are illustrated by the synthesis of γ,γ-hexapeptides **37** and **38**. Complete synthetic description for each product and full characterization of new compounds are provided in ESI.†

γ,γ-Hexapeptides 37 and 38

The MBHA–polystyrene resin was washed for 20 min with a 40% TFA solution in DCM, followed by addition of 20% DIPEA solution in DCM for 3 min. The reaction was monitored by the ninhydrin test. Then, the resin was washed with DMF in order to proceed with attaching the first amino acid. Commercially available proline derivative (2*S*,4*S*)-4-[(9*H*-fluoren-9-yl)-methoxycarbonylamino]-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (3 eq.) was coupled to the resin using 3 equivalents of HBTU and HOBt and 9 equivalents of DIPEA, with DMF as solvent. The reaction was monitored by the ninhydrin test. Then the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). After that, the Fmoc group was cleaved by washing with a 50% solution of piperidine in DMF (2 × 10 min). (1*S*,3*R*)-3-[(9*H*-Fluoren-9-ylmethoxycarbonyl)amino]-2,2-dimethylcyclobutanecarboxylic acid (previously deprotected by reaction of the *tert*-butyl ester derivative **14** with a 40% TFA solution in DCM) was then coupled using HBTU and HOBt as coupling agents in the same proportions as described before. Two more proline residues and 2 more cyclobutane amino acids were coupled in alternation following the same procedure. N^{α}-Proline Boc protecting groups were removed by treatment with TFA in DCM. The excess of TFA was eliminated by washing with a 10% solution of DIPEA in DCM. Then *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine (5 eq.) was reacted with the free amino groups in the presence of Et_3N (9 eq.) in DCM. The reaction was monitored by the chloranil test. Fmoc removal from the N-terminus was achieved by washing the resin with a 50% solution of piperidine in DMF (2 × 10 min). A part of the resin was separated to obtain free γ,γ-hexapeptide **37** and the rest was used to continue with the preparation of **38**.

For the synthesis of **37**, Boc protection of the guanidinium groups was removed by treating the resin with a 40% solution of TFA in DCM. The hexapeptide was cleaved from the resin by washing the resin with MeOH (3 × 1 min), drying it and then treating it with HF in the presence of 10% anisole for 1 h at 0 °C. The crude peptide was purified by semi-preparative HPLC using a non-linear gradient of MeCN and H_2O containing 0.1% of TFA (5% MeCN for 3 min, increased to 18% in 1 min, maintained at 18% for 11 min, increased to 100% MeCN in 1 min, 3 min at 100% in MeCN and finally the original conditions were re-established). The purity of each fraction was verified by

analytical HPLC and MALDI-TOF and showed that the peptide was 97% pure. MS calcd for $C_{39}H_{67}N_{16}O_6$ $[M + H]^+$: 855.54. MALDI-TOF found: 855.48 $[M + H]^+$, 877.47 $[M + Na]^+$, and 893.45 $[M + K]^+$. mp 167–169 °C (from CH_3CN–H_2O); $[\alpha]_D$ = +17 (c = 0.1, CH_3OH). IR (ATR): ν 3187, 2963, 1688, 1658, 1641, 1631, 1611 cm^{-1}. δ_H (360 MHz, CD_3OD) 0.90–1.33 (c.s., 18H), 1.61 (c.s., 3H), 1.93–2.33 (c.s., 12H), 2.47–3.06 (c.s., 10H), 3.59–4.04 (c.s., 6H), 4.48 (c.s., 2H); m/z (ESI): Found, 428.2756 $[(M + 2H)/2]^+$. Calcd for $(C_{39}H_{68}N_{16}O_6)/2$: 428.2748.

The rest of the resin was used to prepare **38**. This part was reacted with the fluorescent label 5(6)-carboxyfluorescein (CF, 5 eq.) using HBTU–HOBt (5 eq.) as coupling reagents, in the presence of Et_3N (10 eq.), followed by washing with piperidine. Then, Boc protection of the guanidinium groups was removed by treating the resin with a 40% solution of TFA in DCM. Finally, the hexapeptide was cleaved from the resin by washing the resin with MeOH (3 × 1 min), drying it and then treating it with HF in the presence of 10% anisole for 1 h at 0 °C. γ,γ-Hexapeptide **38** was then precipitated with cold anhydrous MTBE, filtered, dissolved in an aqueous solution containing acetic acid and then lyophilized. The crude peptide was purified by semi-preparative HPLC using a non-linear gradient of MeCN and H_2O containing 0.1% of TFA (5% MeCN for 3 min, increased to 35% in 1 min, from 35 to 38% in 11 min, increased to 100% MeCN in 1 min, 3 min at 100% in MeCN and finally the original conditions were re-established). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptide was 99% pure. MS calcd for $C_{60}H_{80}N_{16}O_{12}$ $[M + H]^+$: 1216.61. MALDI-TOF found: 1216.40 $[M + H]^+$.

Acknowledgements

The authors acknowledge financial support from Spanish Ministerio de Ciencia e Innovación (grants CTQ2008-00177/BQU, CTQ2010-15408/BQU and SAF2011-30508-C02-01), Generalitat de Catalunya (grant 2009SGR-733) and CIBER-BBN, Networking Centre on Bioengineering, Biomaterials, and Nanomedicine Institute for Research in Biomedicine. They are also grateful to European Union for COST Action CM0803. Time allocated in the Servei de Ressonància Magnètica Nuclear (UAB) is gratefully acknowledged.

References

1 (*a*) T. Hintermann and D. Seebach, *Chimia*, 1997, **50**, 244; (*b*) D. Seebach, S. Abele, J. V. Schreiber, B. Martinoni, A. K. Nassbaum, H. Schild, H. Hennecke, R. Woessner and F. Bitsch, *Chimia*, 1998, **52**, 734; (*c*) D. Seebach, A. K. Beck and D. J. Bierbaum, *Chem. Biodiversity*, 2004, **1**, 1111.

2 For some examples, see: (*a*) D. Seebach, K. Namoto, Y. R. Mahajan, P. Bindschadler, R. Sustmann, M. Kirsch, N. S. Ryder, M. Weiss, M. Sauer, C. Roth, S. Werner, H. D. Beer, C. Munding, P. Walde and M. Voser, *Chem. Biodiversity*, 2004, **1**, 65; (*b*) T. B. Potocky, J. Silvius, A. K. Menon and S. H. Gellman, *ChemBioChem*, 2007, **8**, 917; (*c*) C. Foged, H. Franzyk, S. Bahrami, S. Frokjaer, J. W. Jaroszewski, H. M. Nielsen and C. A. Olsen, *Biochim. Biophys. Acta, Biomembr.*, 2008, **1778**, 2487; (*d*) C. Rueter, C. Buss, J. Scharnert, G. Heusipp and M. A. Schmidt, *J. Cell Sci.*, 2010, **123**, 2190; (*e*) E. Eiriksdottir, K. Konate, U. Langel, G. Divita and S. Deshayes, *Biochim. Biophys. Acta, Biomembr.*, 2010, **1798**, 1119.

3 See for example: P. Claudon, A. Violette, K. Lamour, M. Decossas, S. Fournel, B. Heurtault, J. Godet, Y. Mély, B. Jamart-Grégoire, M.-C. Averlant-Petit, J.-P. Briand, G. Duportail, H. Monteil and G. Guichard, *Angew. Chem., Int. Ed.*, 2010, **49**, 333.

4 See for example: (*a*) M. Lindgren, M. Hällbrink, A. Prochiantz and U. Langel, *TiPS*, 2000, **21**, 99; (*b*) U. Langel, *Cell-Penetrating Peptides in Processes and Applications*, CRC Press Pharmacology and Toxicology Series, Boca Raton, 2002; (*c*) P. Lundberg and U. Langel, *J. Mol. Recognit.*, 2003, **16**, 227.

5 B. L. Davidson and X. O. Breakefield, *Nat. Rev. Neurosci.*, 2004, **4**, 353.

6 (*a*) J. Connor and L. Huang, *J. Cell Biol.*, 1985, **101**, 582; (*b*) M. Foldvari, C. Mezei and M. Mezei, *J. Pharm. Sci.*, 1991, **80**, 10203.

7 F. T. Gentile, E. J. Doherty, D. H. Rein, M. S. Shoichet and S. R. Winn, *React. Polym.*, 1995, **25**, 207.

8 R. Chakrabarti, D. E. Wylie and S. M. Schuster, *J. Biol. Chem.*, 1989, **264**, 15494.

9 For some recent references, see: (*a*) R. Tréhin and H. P. Merkle, *Eur. J. Pharm. Biopharm.*, 2004, **58**, 209; (*b*) S. El Andaloussi, P. Järver, H. Johansson and Ü. Langel, *Biochem. J.*, 2007, **407**, 285.

10 As representative instances on the use of peptoids and unnatural amino acids, see: (*a*) P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinmann and J. B. Rothbard, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97; (*b*) S. Pujals, J. Fernández-Carneado, M. Ludevid and E. Giralt, *ChemMedChem*, 2008, **3**, 296; (*c*) P. A. Wender, W. C. Galliher, E. A. Goun, L. R. Jones and T. H. Pillow, *Adv. Drug Delivery Rev.*, 2008, **60**, 452.

11 (*a*) M. Green and P. M. Loewenstein, *Cell*, 1988, **55**, 1179; (*b*) A. D. Frankel and C. O. Pabo, *Cell*, 1988, **55**, 1189; (*c*) E. Vivès, P. Brodin and B. Lebleu, *J. Biol. Chem.*, 1997, **272**, 16010.

12 (*a*) K. Sadler, K. D. Eom, J.-L. Yang, Y. Dimitrova and J. P. Tam, *Biochemistry*, 2002, **41**, 14150; (*b*) J. Fernández-Carneado, M. J. Kogan, S. Castel, S. Pujals and E. Giralt, *Angew. Chem., Int. Ed.*, 2004, **43**, 1811; (*c*) J. Fernández-Carneado, M. J. Kogan, S. Pujals and E. Giralt, *Biopolymers*, 2004, **76**, 196; (*d*) S. Pujals and E. Giralt, *Adv. Drug Delivery Rev.*, 2008, **60**, 473 and references therein (*e*) I. Geisler and J. Chmielewski, *Chem. Biol. Drug Des.*, 2009, **73**, 39.

13 L. Crespo, G. Sanclimens, B. Montaner, R. Pérez-Tomás, M. Royo, M. Pons, F. Albericio and E. Giralt, *J. Am. Chem. Soc.*, 2002, **124**, 8876.

14 J. Farrera-Sinfreu, L. Zaccaro, D. Vidal, X. Salvatella, E. Giralt, M. Pons, F. Albericio and M. Royo, *J. Am. Chem. Soc.*, 2004, **126**, 6048.

15 J. Farrera-Sinfreu, E. Giralt, S. Castel, F. Albericio and M. Royo, *J. Am. Chem. Soc.*, 2005, **127**, 9459.

16 (*a*) F. Rúa, S. Boussert, T. Parella, I. Díaz-Pérez, E. Giralt, V. Branchadell and R. M. Ortuño, *Org. Lett.*, 2007, **9**, 3643; (*b*) E. Torres, E. Gorrea, E. Da Silva, P. Nolis, V. Branchadell and R. M. Ortuño, *Org. Lett.*, 2009, **11**, 2301; (*c*) E. Torres, E. Gorrea, K. K. Burusco, E. Da Silva, P. Nolis, F. Rúa, S. Boussert, I. Díez-Pérez, S. Dannenberg, S. Izquierdo, E. Giralt, C. Jaime, V. Branchadell and R. M. Ortuño, *Org. Biomol. Chem.*, 2010, **8**, 564; (*d*) E. Gorrea, E. Torres, P. Nolis, E. Da Silva, D. B. Amabilino, V. Branchadell and R. M. Ortuño, *Chem.–Eur. J.*, 2011, **17**, 4588; (*e*) S. Celis, E. Gorrea, P. Nolis, O. Illa and R. M. Ortuño, *Org. Biomol. Chem.*, 2012, **10**, 861.

17 J. Aguilera, A. G. Moglioni, G. Y. Moltrasio and R. M. Ortuño, *Tetrahedron: Asymmetry*, 2008, **19**, 302.

18 D. Fernández, E. Torres, F. X. Avilés, R. M. Ortuño and J. Vendrell, *Biorg. Med. Chem.*, 2009, **17**, 3824.

19 R. Gutiérrez-Abad, D. Carbajo, P. Nolis, C. Acosta-Silva, J. A. Cobos, O. Illa, M. Royo and R. M. Ortuño, *Amino Acids*, 2011, **41**, 673.

20 M. Pätzel, M. Sanktjohanser, A. Doss, P. Henklein and G. Szeimies, *Eur. J. Org. Chem.*, 2004, 493.

21 (*a*) S. Robinson and E. J. Roskamp, *Tetrahedron*, 1997, **53**, 6697; (*b*) G. J. Gabriel, A. E. Madkour, J. M. Dabkowski, C. F. Nelson, K. Nüsslein and G. N. Tew, *Biomacromolecules*, 2008, **9**, 2980.

22 R. Fischer, O. Mader, G. Jung and R. Brock, *Bioconjugate Chem.*, 2003, **14**, 653.

23 J. Farrera-Sinfreu, M. Royo and F. Albericio, *Tetrahedron Lett.*, 2002, **43**, 7813.

24 E. A. Goun, T. H. Pillow, L. R. Jones, J. B. Rothbard and P. A. Wender, *ChemBioChem*, 2006, **7**, 1497.

25 R. Abes, H. M. Moulton, P. Clair, S.-T. Yang, S. Abes, K. Melikov, P. Prevot, D. S. Youngblood, P. L. Iversen, L. V. Chernomordik and B. Lebleu, *Nucleic Acids Res.*, 2008, **36**, 6343.